

Attenuation of cisplatin-induced acute kidney injury by N-(2-Hydroxyphenyl) acetamide and its gold conjugated nano-formulations in mice

Abdul Kadir¹, Sadaf Ibrahim¹, Syed Nudrat Nawaid Shah², Zara Aslam³,
Rehan Ahmed Siddiqui⁴, Mirza Tasawer Baig^{1*}, Muhammad Raza Shah³, Talat Mirza⁴,
Shabana Usman Simjee^{3,5} and Adeel Arsalan¹

¹Faculty of Pharmacy, Ziauddin University, Karachi, Pakistan

²Department of Anatomy, Ziauddin University, Karachi, Pakistan

³HEJ Research Institute of Chemistry, International Center for Chemical and Biological Science, University of Karachi, Pakistan

⁴Department of Research, Ziauddin University, Karachi, Pakistan

⁵Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Science, University of Karachi, Karachi, Pakistan

Abstract: The attenuation of cisplatin-induced acute kidney injury (AKI) in mice by N-(2-hydroxyphenyl) acetamide (NA-2) and NA-2-conjugated gold nanoparticles (NA2-AuNPs) was investigated. Male BALB/c mice (n = 54) were divided into nine groups having six animals in each group. Animals in groups 3-9 were pre-treated for 5 days with test compounds, whereas, animals in group 1 and 2 received normal saline. On day 4, animals in groups 2, 3, 4, 5, 6 and 9 were given single intra-peritoneal injection of CP at the dose of 5 mg/kg. After 72 hours of CP injection, all animals were sacrificed. Blood was collected for serum urea and creatinine estimation, and kidneys were harvested for histo-pathological examinations and qPCR studies for nuclear factor- κ B p50, (NF κ B); inducible nitric oxide synthase (iNOS); hemeoxygenase-1 (HO-1); and interleukin-6 (IL-6). NA-2 and NA2-AuNPs was observed to decrease the serum urea and creatinine levels. Both the test compounds reduced kidney injury damage score and improved histological architecture in the treated animals in dose dependent manner. Furthermore, the mRNA expressions of NF κ B p50, iNOS and IL-6 genes were down-regulated, and HO-1 gene was up-regulated in the animals treated with the test compounds. It is concluded that NA-2 and NA2-AuNPs attenuates CP-induced AKI in mice models through anti-inflammatory and anti-oxidant mechanisms.

Keywords: Acute kidney injury, gold nanoparticles, N-(2-hydroxyphenyl) acetamide, cisplatin.

INTRODUCTION

Acute Kidney Injury (AKI), which can be a consequence of many clinical conditions, usually present as anuria and increase in serum creatinine levels (Mehta *et al.*, 2007). It is usually under recognized and could lead to serious repercussion like chronic kidney disease (Uchino *et al.*, 2005). Recent studies have suggested that the incidence of AKI varies around 23.8 cases per 1000 discharge and it has increased dramatically in the last few decades (Xue *et al.*, 2006). Clinical conditions like inflammation, ischemia, renal obstruction or drug induce injury are the common causes of AKI (Jha *et al.*, 1992). Although, many drugs cause Nephrotoxicity but these nephrotoxic effects can be minimized or prevented with prompt intervention (Schetz *et al.*, 2005). Nephrotoxicity of chemotherapeutic agents is well reported and recognized (Perazella & Moeckel, 2010). Infact 60% of patients receiving chemotherapeutic agents are reported to have some degree of kidney disease (Sahni *et al.*, 2009). Among these nephrotoxic chemotherapeutic agents, a platinum based drug Cisplatin (CP) is prescribed in many

types of cancers such as testicular cancer, ovarian cancer, cancers of head and neck region (Desoize & Madoulet, 2002).

The nephrotoxic effects of CP are the cause of great concern as it greatly affects the morbidity and mortality of patients. It is reported to cause apoptosis and necrosis of many components of nephron including glomeruli but mostly affecting the tubular epithelium of proximal convoluted tubules (Pabla & Dong, 2008). These damaging effects of CP on proximal convoluted tubules are reported to be caused by many mechanisms i.e. apoptosis, autophagy, dysregulation of mitogen activated protein kinase pathway and cell to cell cycle proteins and DNA damage (Ozkok & Edelstein, 2014). In recent times, many advances are made in cancer treatment which has dramatically changed the landscape of oncology. Better therapeutic agents with better results and less adverse effects have been made which have greatly benefited the patients (Winer *et al.*, 2009). However, new quests are being planned to explore new therapeutic compounds which could reduce the injurious effects of these vital chemotherapeutic drugs.

*Corresponding author: e-mail: mirzatasawerbaig@gmail.com

Recently, it has been reported that N-(2-hydroxyphenyl) acetamide (NA-2) has a kidney protective role in AKI induced by glycerol in animal models (Siddiqui *et al.*, 2019). NA-2 is a salicylic acid derivative having anti-inflammatory action reported *in vivo* model of arthritis (Perveen *et al.*, 2013). Its apoptosis inducing properties are also reported *in vitro* model of human glioblastoma cell line (Hanif *et al.*, 2014). In this study, NA-2 is selected to assess its protective effect in animal model of CP-induced AKI. Furthermore, gold nano-conjugation of NA-2 (NA2-AuNPs) was also used as the nano-formulations increase bioavailability, deliver the drug to specific target site and decrease the cellular toxicity (Martis *et al.*, 2012).

MATERIALS AND METHODS

Animal protocols

54 male Balb/c mice, weight 25±30 gm were selected for experimental study. Animals were procured from Animal House, HEJ Research Institutes of Chemistry, University of Karachi. The animals were kept in the sterile plastic cage in an alternating 12 hours light/dark cycle at a temperature of 25±2°C for acclimatization and dosing of animals animal house of Ziauddin University. Duration of the study was 9 months. An approval has been taken from Animal Ethics Committee of Ziauddin University before start the experiment (Protocol No. 2019-003). The animals were kept on same and uniform diet.

Treatment regimen

Mice were divided into nine groups, each groups contains 6 mice i.e. normal control (Group 1 $n_1=6$); CP (Group 2, $n_2=6$) received cisplatin 5mg/kg; CP + NA-2 25mg/kg (Group 3 $n_3=6$) CP + NA-250mg/kg, (Group 4 $n_4=6$) CP + NA2-AuNPs 15mg/kg (Group 5 $n_5=6$); CP + NA2-AuNPs 25mg/kg (Group 6 $n_6=6$); NA-2 only 50 mg/kg (Group 7 $n_7=6$); NA2-AuNPs only 25 mg/kg (Group 8 $n_8=6$); and CP + Ascorbic Acid (Group 9 $n_9=6$). All animals were pre-treated with *intra-peritoneal* administration of the test compounds for 5 days except groups 1 and 2 which received normal saline. On day 4, animals in groups 2, 3, 4, 5, 6 and 9 were given single *intra-peritoneal* injection of CP at the dose of 5 mg/kg. 72 h later of CP administration, animals were dissected humanely under general anesthesia. Blood was collected for serum urea and creatinine estimation, and kidneys were harvested for histo-pathological examinations and RT qPCR studies.

Synthesis of N-(2-hydroxyphenyl) acetamide Coated Gold Nanoparticles (NA-2-AuNPs)

N-(2-hydroxyphenyl) acetamide conjugated gold nanoparticles were synthesized by NaBH₄ reduction method. Briefly, aqueous solution of N-(2-hydroxyphenyl) acetamide (2mg/mL) was prepared in deionized water followed by sonication for 30 minutes. Stock solution of tetrachloroauric (III) acid trihydrate (HAuCl₄) solution (0.1mM) was prepared in 50 mL. A 5

mL of aqueous solution of tetrachloroauric (III) acid trihydrate was taken in a glass vial having a magnetic stir bar. 5mL solution of N-(2-hydroxyphenyl) acetamide (2 mg/mL) was added drop wise upon stirring at 200 rpm. After 10 minutes, 10 μ L of freshly prepared aqueous solution of NaBH₄ (5mM) was added drop wise into the solution. The color of the reaction mixture changed from pale white to deep red indicating the formation of N-(2-hydroxyphenyl)acetamide coated gold nanoparticles which was further confirmed by UV-visible spectroscopy (Siddiqui *et al.*, 2019).

Serum urea and creatinine estimation

For the estimation of serum urea and creatinine levels, blood samples were collected by cardiac puncture technique. The serum levels of urea and creatinine was measured by spectrophotometric method using Microlab 300 (ELI Tech Group) (Siddiqui *et al.*, 2019).

Histological examination of kidney tissues

After animal dissection, kidney tissue samples were collected and fixed in Bouin's fixative for 4 h. Later on, washing was done with de-ionized water. Tissue sample were kept in 70% isopropanol for overnight at room temperature. Isopropanol was used for dehydration in ascending concentration. Clearing was done by xylene and specimens were then embedded in paraffin. 5 μ m thick tissue sections were made and hemato-xylins and eosin (H&E) and periodic acid Schiff (PAS) stains were performed. Slides were observed under Nikon Ts2R-FL inverted microscope. Nikon Elements D software was used to calculate damaged area (Siddiqui *et al.*, 2019).

mRNA expressions of NF κ B p50, iNOS, HO-1 and IL-6 by RTqPCR

The mouse kidney tissues were homogenized and mRNA was isolated using TRIzol reagent (Life Technologies, USA). cDNA was synthesized by Revert Aid first strand cDNA synthesis kit (Fermentas, USA). Previously, published primers were used. qPCR cycles were set at 94°C for 5 minutes, followed by 40 cycles at 94°C for 5 seconds, 60°C for 30 seconds and 94°C for 15 seconds. GAPDH was used to normalize mRNA expressions, and the expression levels were calculated using the 2^{- $\Delta\Delta$ Ct} method (Siddiqui *et al.*, 2019).

STATISTICAL ANALYSIS

Data was evaluated using SPSS version 21. Means and standard deviation were calculated. Statistical significance between groups was evaluated using t-test and p value <0.05 was considered statistically significant.

RESULTS

Characterization of N-(2-hydroxyphenyl) acetamide coated gold nanoparticles (NA-2-AuNPs)

Synthesis of N-(2-hydroxyphenyl) acetamide coated gold nanoparticles (NA-2-AuNPs) was carried out by chemical

reduction method. The synthesized nanoparticles showed the surface Plasmon resonance (SPR) band at 540 nm (Santhoshkumar *et al.*, 2017). Which confirmed the formation of N-(2-hydroxyphenyl) acetamide coated gold nanoparticles (NA-2-AuNPs) as shown in fig. 1A.

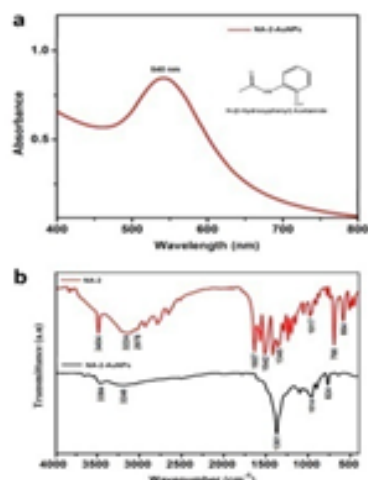


Fig. 1: (a) Chemical structure of NA-2 and UV-Visible spectrum of NA-2-AuNPs. (b) FTIR spectra of NA-2 (red trace) and NA-2-AuNPs (black trace)

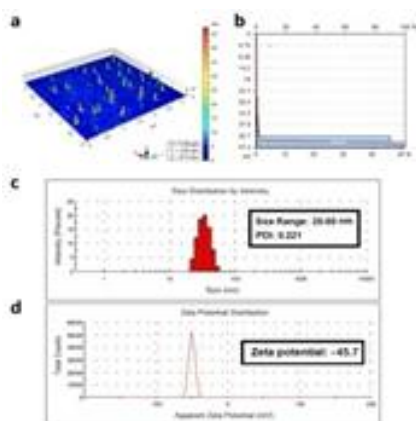


Fig. 2: AFM images of NA-2-AuNPs (a) 3D-height image and (b) average particle height distribution graph. Dynamic light scattering (DLS) analysis of NA-2-AuNPs (c) average particle size distribution histogram and (d) zeta potential graph

FTIR analysis was performed for the evaluation of effective coating of N-(2-hydroxyphenyl) acetamide over gold nanoparticles. The comparative FTIR spectrum of N-(2-hydroxyphenyl) acetamide (red trace) and N-(2-hydroxyphenyl) acetamide coated gold nanoparticles (NA-2-AuNPs) (black trace) are given in fig. 1B. The FTIR spectra of N-(2-hydroxyphenyl) acetamide coated gold nanoparticles (NA-2-AuNPs) showed predominant shifts in stretching vibrations of N-H and O-H bonds which shifted and appeared at 3384 cm^{-1} and 3246 cm^{-1} . This might be due to the involvement of these bonds in the successful coating of N-(2-hydroxyphenyl)

acetamide over the gold nanoparticles surface since it has been reported that phenolic and nitrogen containing groups conjugate and stabilize nanoparticles (Kaur & Kumar, 2019; Ul Ain *et al.*, 2019). Strong stretching vibrational peak appearing at 1361 cm^{-1} corresponds to the C-N bond (aromatic) while the peak at 1014 cm^{-1} corresponds to the stretching vibration of C-O bond. The peak shifting indicates the effective conjugation of N-(2-hydroxyphenyl) acetamide over the gold nanoparticles.

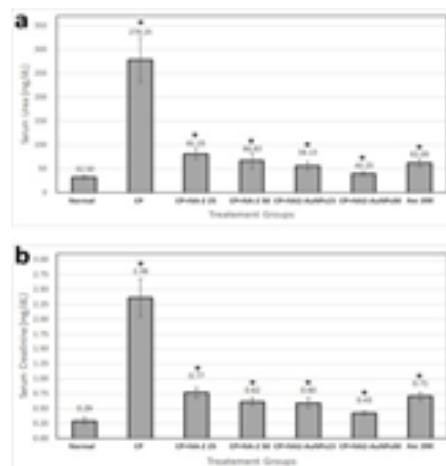


Fig. 3: Serum urea and creatinine levels. Serum urea (a) and creatinine (b) levels were significantly elevated in cisplatin-induced AKI group in comparison with the normal control. The levels were significantly decreased in all groups treated with the test compounds in comparison with the CP group. (* $p < 0.001$)

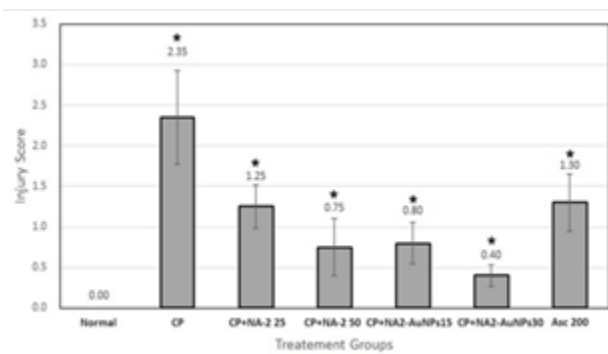


Fig. 4: Kidney injury score. Kidney injury score was significantly increased in cisplatin-induced AKI group in comparison with the normal control. The injury score was significantly reduced in all groups treated with the test compounds. It can be noted that very low score was seen in the animals treated with NA-2-AuNPs at the dose of 25 mg/kg. (* $p < 0.001$)

Atomic force microscopy was used for the evaluation of shape and morphological analysis of N-(2-hydroxyphenyl) acetamide coated gold nanoparticles (NA-2-AuNPs). The results are given in fig. 2. The AFM result exhibited the uniformly distributed pointed shaped nanoparticles as shown in fig. 2A. The average height of

nanoparticles is in the range of 42-47 nm which is evident by particle height distribution graph in fig. 2B.

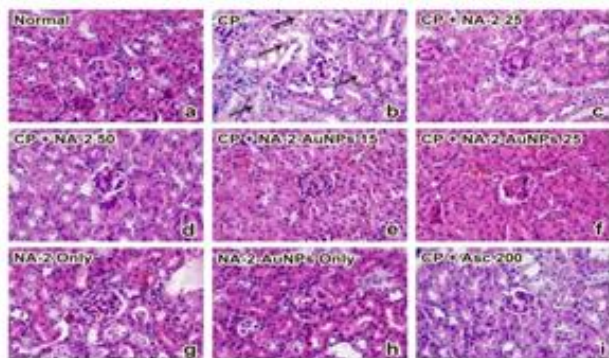


Fig. 5: H&E stained images showing cortex of kidney sections. Image 'a' is showing normal kidney structure whereas image b represents CP treated group. The arrow shows damaged tubular cells. Images c and d are of CP+NA-2 treated groups with different doses i.e. 25 mg/kg and 50 mg/kg respectively showing remarkable decrease in tubular damage. Images e and f are of CP+NA2-AuNPs treated groups with different doses i.e. 15mg/kg and 25mg/kg respectively showing almost complete protection. Images g and h are of NA-2 only and NA2-AuNPs only treated group showing no kidney toxicity by the test compounds. Image i represents ascorbic acid treated group which was used as a positive control. Magnification, 400x.

Zeta sizer analysis was carried out for the determination of average particle size of the synthesized nanoparticles. fig. 2C shows the size distribution graph of NA-2-AuNPs. The average particle size of nanoparticles was found in the range of 25-60 nm having poly dispersity index (PDI) of 0.221. The PDI value indicates the uniformity and extent of dispersion of nanoparticles. PDI values less than 0.3 correspond to monodisperse nanoparticles having uniform size distribution. Zeta potential of NA-2-AuNPs was found to be -45.7 mV. Negative zeta potential value corresponds to the higher stability of nanoparticles due to the electrostatic repulsion between negative charges which prevents aggregation (Wrótniak-Drzewiecka *et al.*, 2014).

NA-2 and NA2-AuNPs reduced serum urea and creatinine levels

Fig. 3A and B demonstrates a significant rise in the levels of serum urea and creatinine respectively in animals of CP group as compared to the normal control (p value <0.001) which exhibit the nephrotoxic effects of CP. Statistically significant (p value <0.001) improvements in the serum levels of urea and creatinine in groups 3, 4, 5 and 6 were observed which represents the mitigating effects of NA-2 and its gold nanoparticles conjugation. The mitigating effect on the urea and creatinine levels increased with the dose of NA-2 and NA2-AuNPs.

Group7 which was treated with ascorbic acid was taken as positive controls. Group 6 in which mice were treated with NA2-AuNPs at a dose of 25mg/kg showed the best results which might suggest the better efficacy and bioavailability of the drug when conjugated with gold nanoparticles.

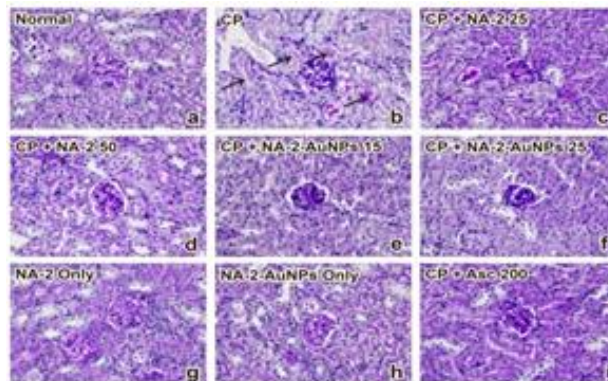


Fig. 6: PAS stained images showing brush borders of proximal convoluted tubules in the cortex of kidney. Image a is showing normal kidney tubules and intact brush borders whereas image b is of CP treated group exhibiting noticeable damaged brush borders of proximal convoluted tubules (arrows). Images c and d are of CP+NA-2 treated groups with different doses i.e. 25 mg/kg and 50 mg/kg respectively showing decrease in brush border destruction. Images e and f are of CP+NA2-AuNPs treated groups with different doses i.e. 15 mg/kg and 25 mg/kg respectively showing almost complete protection of brush borders. Images g and h are of NA-2 only and NA2-AuNPs only treated group showing no kidney toxicity by the test compounds. Image i represents ascorbic acid treated group which was used as a positive control. Magnification, 400x.

NA-2 and NA2-AuNPs reduced kidney damage scores

Fig. 4 showed a statistically significant rise (p value <0.001) in the injury scores in animals of group 2 as compared to group 1 which demonstrate the nephrotoxic effects of CP. Statistically significant (p value <0.001) improvements in the injury scores in group 3, 4, 5 and 6 were observed which represents the ameliorating effects of NA-2 and its gold nanoparticles conjugation. The ameliorating effect on the injury scores increased with the dose of NA-2 and NA2-AuNPs. Group7 which was treated with ascorbic acid was taken as controls. Best results were obtained in the animals treated with NA2-AuNPs at a dose of 25mg/kg suggesting better bioavailability and efficacy of the drug when conjugated with gold nanoparticles.

NA-2 and NA2-AuNPs improved histological architecture

Figs. 5 and 6 represent H&E and PAS stained photomicrographs of kidney tissues respectively. Fig. 5A showed normal structure of renal cortex with normal

architecture of renal corpuscles and tubules. However, fig. 5B showed excessive epithelial vacuolization, glomerular atrophy, the damaged tubular architecture particularly in proximal tubules caused by CP. Figs. 5C and D showed progressive improvements in epithelial vacuolization, protein cast in the tubular lumen and in the extent of damage of proximal tubules with the dosage of NA-2 demonstrating its protective effects. Figs. 5E and F showed more marked improvements in epithelial vacuolization, damage to proximal convoluted tubules when the gold nanoparticles conjugated drug is used. Figs. 5G and H showed the intact nephron architecture when the animals were treated with NA-2 only and NA2-AuNPs respectively. fig. 5I shows kidney sections of ascorbic acid treated group which was used as positive control.

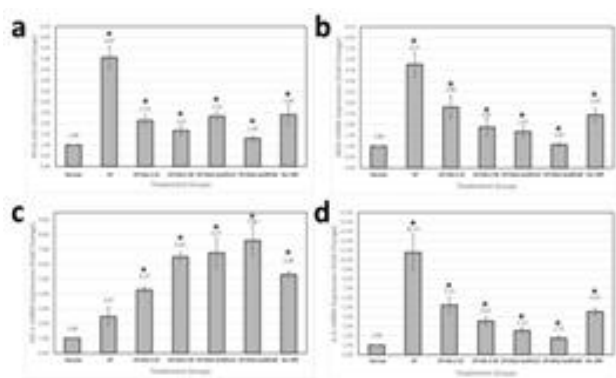


Fig. 7: mRNA expressions of iNOS, NF- κ B, HO-1 and IL-6. NA-2 and NA2-AuNPs down-regulate the mRNA expressions of NF- κ B p50 (a), iNOS (b) IL-6 (d) while both up-regulate the mRNA expressions of HO-1 (c). (* $p < 0.001$)

Fig. 6A showed normal brush borders of proximal convoluted tubules (PCT). However, fig. 6B showed extensive damage in the brush borders of PCT with increased protein cast deposition in the tubular lumen caused by CP. Figs. 6C and D showed lesser extent of damage in the brush border of PCT in the NA-2 treated groups with progressive improvement with the dosage of NA-2 representing its mitigating effects on the microvilli of tubular epithelial cells. Figs. 6E and F showed marked decrease in microvilli of tubular epithelial cells of PCT when the gold nanoparticles conjugated drug were used. Figs. 6G and H showed the intact nephron architecture when the mice were treated with NA-2 only and NA2-AuNPs respectively. fig. 6I showed kidney sections of ascorbic acid treated group which was used as positive control.

NA-2 and NA2-AuNPs down-regulate the mRNA expressions of NF κ B p50, iNOS and IL-6 and up-regulate HO-1 genes

Fig. 7 represents real time PCR analysis of NF κ B p50, iNOS, HO-1 and IL-6 mRNA. A statistically significant

rise in mRNA expressions of inflammatory markers iNOS, NF κ B and IL-6 in figs. 7A, B and D can be noted between normal and CP group which demonstrate increased inflammatory response after CP injection due to AKI. Figs. 7A, B and D showed progressive improvements in the mRNA expressions of iNOS, NF κ B and IL-6 in group 3 and 4, treated with increasing doses of NA-2. Progressive mitigating effects of NA2-AuNPs were observed with increasing dose. Least inflammatory mRNA expressions of NF κ B, iNOS and IL-6 were observed in NA2-AuNPs group at the dose of 25mg/kg demonstrating the better efficacy and bioavailability. However, HO-1 which is a tubular protective protein, whose expression increased in response to inflammation, shows progressive significant increase in its mRNA expression in all groups (fig. 7C). The result suggested a marked increase in protective anti-inflammatory activity of NA-2 and NA2-AuNPs.

DISCUSSION

Cisplatin (CP) is among those drugs which are reported to have nephrotoxic effects. Though, it is prescribed for the treatment of large variety of tumors involving lungs, testes, ovaries, urinary bladder, head and neck (Kodama *et al.*, 2014). Its use is restricted by its nephrotoxic potential as even a single dose of CP (50-100mg/m²) may results in nephrotoxic effects in one third of the patients (Lebwohl *et al.*, 1998, Shiraishi *et al.*, 2000). Identifying better ways to minimize the nephrotoxic effects of CP could greatly improve the morbidity and mortality of the patients. Therefore, animal model of CP-induced AKI was used to study the mitigating effects of NA-2 and its gold nanoparticles conjugation on it.

In this study, the protective effects of NA-2 and NA2-AuNPs are elucidated on mouse kidney and it was found that the test compound and its nano-conjugation effectively prevented and reduces the CP-induced AKI. Findings of kidney injury induced by CP are in agreement with the previous reported findings which suggest that CP causes damage to many components of nephron including glomeruli, tubular epithelium and blood vessels (Pabla & Dong, 2008). In the present study, the tested compounds effectively reduced serum urea and creatinine levels, reduced the kidney injury scores, decreases the epithelial vacuolization caused by CP and improved the tubular damage by preventing the insult to the architecture of microvilli forming the brush borders of tubular epithelium. These effects may be due to the anti-inflammatory effects of NA-2 (Perveen *et al.*, 2013). In this study, NA-2 is also incorporated with gold nanoparticles. It is found that nanoparticles conjugation lead to dramatic increase in kidney protective activity of NA-2. This could be due to greater specific site binding potential of nanoparticles, decreased cellular toxicity, more bioavailability associated with nanoparticles (Martis *et al.*, 2012).

The present study also investigated the likely mechanisms involved for this ameliorating effect of NA-2 by evaluation of mRNA expressions of inflammatory markers such as NF- κ B, iNOS and IL 6. NF- κ B is a protein which controls the duration and degree of inflammatory process by multiple mechanisms (Lawrence, 2009; Hayden *et al.*, 2008). Similarly, iNOS regulates inflammatory cytokines i.e. nitric oxide (Mungrue *et al.*, 2004) and IL-6 levels are increased during inflammation (Hack *et al.*, 1989).

A significant increase in mRNA expressions of iNOS, NF- κ B and IL-6 was found in cisplatin (CP) group compared to the normal group which signifies increased injury due to inflammatory processes. A progressive decline in mRNA expressions of iNOS, NF- κ B and IL-6 observed with the treatment of NA-2 and its gold nanoparticles conjugates suggests enhanced anti-inflammatory activity by NA-2 and NA2-AuNPs as NF- κ B, iNOS, and IL 6 are the key inflammatory mediators which regulates inflammation (Chander *et al.*, 2003).

We also evaluated HO-1 mRNA expression, which is a microsomal enzyme that catalyzes the degradation of heme into biliverdin, iron, and carbon monoxide (Agarwal *et al.*, 2000). HO-1 is known to be activated in the kidney by CP treatment (Agarwal *et al.*, 1995). In this study, a significant progressive increase in the mRNA expression of HO-1 is found in groups treated with NA-2 and NA2-AuNPs. These findings suggest kidney protective mechanisms as reported in the literature that stress factors increases HO-1 expression which has immune modulating and anti-inflammatory functions (Piantadosi *et al.*, 2011; Gozzelino *et al.*, 2010).

CONCLUSION

Present study findings suggest that NA-2 has a nephron protective function. It can effectively reduce and prevent AKI caused by CP. It inserts its effects by altering inflammatory and oxidative process. Conjugation of NA-2 with gold nanoparticles dramatically increases its anti-inflammatory and anti-oxidant activities thus greatly influence its renal protective potential. Further studies on this compound are strongly recommended.

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